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14. ABSTRACT
The goal of the experiments was to test if the acetylenes could be used to suppress the in vivo growth of P210Bcr-Abl dependent cell lines. The work completed during the funding showed that the acetylene compound K1P, which had been shown to suppress the in vitro growth of CML cells which are resistant to imatinib, and to inhibit p210Bcr-Abl dependent in vitro CML cell growth at the nanomolar concentrations when combined with imatinib, could also suppress the in vivo growth of a P210Bcr-Abl dependent cell line (Baf-1P210Bcr-Abl).. These studies also showed that the medium in which the drugs were dissolved must contain 0.1% ethanol to increase the solubility sufficiently to suppress in vivo P210Bcr-Abl dependent cell growth. Finally, the presence of serum in the medium used to expose the drugs to the P210Bcr-Abl dependent cells blocked the suppressive activity of the drugs despite the presence of 0.1% ethanol. These data suggested that chemical functionalities which could improve the bioavailability of the drugs in the presence of serum needed to be added to the ester side chain of the acetylene compounds. This work once completed will enable further in vivo toxicity and efficacy studies to proceed.

15. SUBJECT TERMS
DML, kinases, p210BCR-Ab, imatinib

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I. TABLE OF COMPLETION OF STATEMENT OF WORK

Goal in Statement of Work	Section Number of Progress Report which Pertains to SOW Goal
Specific Aim #1	**Completed**
In vitro effect of test drugs on in vivo growth.	
Task 1	**Completed**
Task 2	**Completed**
Task 3	**Completed**
Task 4	**Completed**
Task 5	**Completed**
Task 6	**Completed**
Task 7	**Completed**
Specific Aim #2	
Study whether test drugs are toxic to the leukemic cells or the normal bone marrow repopulating cells.	
Task 1	**Not Completed due to results in SA#1**
Task 2	**Not Completed due to results in SA#1**
Task 3	**Not Completed due to results in SA#1**
Task 4	**Not Completed due to results in SA#1**
Task 5	**Not Completed due to results in SA#1**
Task 6	**Not Completed due to results in SA#1**
Specific Aim #3	
Test the toxicity of the test drugs on normal tissues.	
Task 1	**Not Completed due to results in SA#1**
Task 2	**Not Completed due to results in SA#1**
Task 3	**Not Completed due to results in SA#1**
Task 4	**Not Completed due to results in SA#1**
Task 5	**Not Completed due to results in SA#1**
Task 6	**Not Completed due to results in SA#1**

Comment: Results in SA #1 showed that the drugs were capable of suppressing in vivo growth of P210Bcr-Abl positive cell lines. The completion of the experiments required to demonstrate this required the entire year of support. During the course of this work, it was found that the inhibitory activity of these drugs was blocked by serum. Thus, it became clear that further structural modification of these drugs with chemical functionalities which would improve their bioavailability in the presence of serum was going to be necessary prior to testing of Specific Aims #2 and #3. We are planning structure activity studies of these drugs once they are modified to increase their bioavailability.

II. INTRODUCTION

Imatinib was shown early on to suppress the level of the circulating myeloid cell mass in most patients with chronic phase CML and a lower fraction of patients with accelerated or acute phase CML. At least 24 different point mutations of the p210Bcr-Abl tyrosine specific protein kinase were found to be associated with the acquisition of resistance to imatinib (1-5). In addition, other mechanisms of resistance were found, other than point mutations, such as overexpression of the p-glycoprotein. The point mutations were found in a small number of patients with primary resistance to imatinib at diagnosis, and a much larger number of patients with acquired resistance at the time of emergence of resistance to imatinib in accelerated and acute phase patients.

Thus, a major priority was to find additional drugs which could be used to treat CML patients in whom resistance to imatinib was acquired or was present at diagnosis. Several drugs have been reported to show suppressed populations of CML cells which display resistance to imatinib (6). Both preclinical and clinical data are available on drugs which inhibit the p210Bcr-Abl kinase both in the activated and inactive states (7). The combination of one of these drugs with imatinib reduce the rate of acquisition of point mutations but does not suppress this process completely. Thus, there is a need for additional compounds which suppress p210Bcr-Abl dependent cell growth, such as those described in this report.

These newer drugs are being tested clinically to determine whether they can suppress the circulating CML myeloid cell mass in patients whose CML cells are not responsive to imatinib. Eventually, such drugs may be combined at diagnosis with imatinib to reduce the probability of evolution of imatinib resistance, especially in patients with accelerated and acute phases of CML, or chronic phase CML patients with adverse clinical features, who are therefore considered to be at increased probability of evolution of resistance to imatinib.

We have identified two groups of compounds which inhibit proliferation of the 32Dtetp210Bcr-Abl cell line under conditions in which cell growth is dependent on the Bcr-Abl protein kinase, in the absence of added growth factors such as IL-3. When the inhibitory effects of acetylene-derived lead drugs (AC22, AC19 and K1P) on the growth of the 32Dtetp210Bcr-Abl cell line were tested in the absence of serum in the low cell density growth inhibition assay, significant inhibition was seen at the 100 nanomolar level. When the same drugs were tested for suppression of growth of the 32Dtetp210Bcr-Abl cell line at high cell density in the presence of serum, the concentration of drugs required for significant growth inhibition is in the 1 micromolar range.

Interestingly, when individual acetylene compounds AC22 and K1P are combined with imatinib in the absence of serum, they suppress the proliferation of the 32Dtetp210Bcr-Abl cells at the 10 nM range. In addition, the inhibitory activity of the combination of AC22 or K1P with imatinib is extremely selective for p210Bcr-Abl dependent growth since no inhibition is seen on the 32D cell line in the low cell density proliferation assay. Similarly, the furan derived compounds are also extremely selective and potent inhibitors of the growth of the 32Dtetp210Bcr-Abl cell line at the 10 nM concentration level.

We also studied the effect of the acetylenes on the growth of two imatinib-resistant cell lines that overexpress Bcr-Abl. These compounds showed a higher inhibitory power on the Baf/BCR-ABL-r than on the LAMA84-r cell line. This may be due to the fact that, in addition to Bcr-Abl overexpression, LAMA84-r, but not Baf/BCR-ABL-r, also overexpresses the MDR1 p-glycoprotein. Whether the acetylene compounds are substrates of this pump protein has not yet been proven. In future work, it will also be important to test the inhibitory effect of the furans and acetylenic compounds on cell lines in which the resistance to imatinib is due to the acquisition of point mutations in the p210Bcr-Abl kinase. This latter mechanism is the most common mechanism through which imatinib resistance is acquired in patients (6-7).

It is remarkable that two classes of such good compounds were identified from so few candidates. Our screening strategy was designed to identify compounds which would inhibit proliferation of the 32Dtetp210Bcr-Abl protein kinase dependent cells at any point downstream of the complex pathways emanating from the action of the p210Bcr-Abl kinase. It is thus possible that this strategy increased the chances of finding compounds which were inhibitory to imatinib-resistant cell lines.

There are several unique features of the acetylenes and furans which distinguish them from the other CML inhibitory drugs already reported (8-12), and which suggest that they may play a unique clinical role.

1. The acetylene AC22 has been shown not only to suppress the growth of CML cells which are resistant to imatinib, but also to inhibit p210Bcr-Abl dependent cell growth at the nanomolar concentrations when combined with imatinib.
2. The level of inhibition of the combination of AC22 and imatinib is greater than the sum of the inhibitory effects of AC22 and imatinib when used separately.
3. The combination of AC22 and imatinib is totally non inhibitory to the non-p210Bcr-Abl dependent cell growth at the 10 nanomolar level.
4. The acetylenes AC22 and K1Pare inhibitory to imatinib resistant cells.
5. The furans (eg A103 and A105) are inhibitory to p210Bcr-Abl dependent cell growth at the 10 nanomolar concentration at which these drugs do not inhibited cell growth which is not p210Bcr-Abl dependent.
6. The acetylene AC19 is inhibitory to p210Bcr-Abl dependent cell growth at the 1 micromolar level at which level the drug is totally non inhibitory to the p210Bcr-Abl independent cell growth.

Thus, the acetylenes: AC22, K1P and AC19 as well as the Furans A103 and A105 have qualities which distinguish them from previously reported CML cell inhibitory drugs and which suggest that they may be very useful when combined with imatinib therapy which is started early in chronic phase in patients with clinical features suggesting a high likelihood of recurrence. In addition, the experimental results suggest that these drugs may be useful for the treatment of patients in whom resistance to imatinib has already emerged.

Our studies showed that the acetylenic compounds K1P and AC22 also inhibit Src and the Src-related kinases Lyn and Fyn. This is a very interesting result in light of the recent report that the Lyn protein kinase is overexpressed in imatinib resistant cell lines K-562-R (13). The Lyn kinase has been reported to be involved in the response of the cell to DNA damaging agents such is the Abl kinase (13). This suggests that these compounds might be acting at multiple different targets downstream of p210Bcr-Abl.

In addition, the use of a synthetic strategy to generate the low-complexity combinatorial libraries of compounds which maintains the chirality of the chemical functionalities on the two types of molecular scaffolds (linear-acetylenes or planar cyclic-furans) may have reduced the number of candidates needed to identify potent and selective inhibitors of the 32Dtetp210Bcr-Alb cell line.

Another remarkable feature that was observed among the acetylene derived compounds from our libraries is that some of them suppress imatinib-resistant cell lines. Such compounds could conceivably be of value when used together with imatinib for the initial treatment of CML, since the probability of the evolution of resistance to a combination might be lower than with single agent therapy.

The study of these acetylenic and furan compounds prior to the initiation of the period of funding has been restricted to in vitro work. Further studies are required to characterize these compounds at the pre-clinical level

before they can be considered for introduction into the clinic. We proposed in the DOD CML application to initiate studies to test whether these drugs could affect P210Bcr-Abl dependent growth and survival pathways in a way which would reduce the growth of cell lines which are dependent on P210Bcr-Abl for growth and survival. Following the studies outlined in the CML DOD application, we plan to utilize side chains for the addition of functional groups which could increase bioavailability and improve the pharmacokinetics.

III. BODY

The studies proposed in this research grant were designed to extend the already completed in vitro studies. The acetylenes K1P and AC22 suppress the P210bcrabl dependent growth of the 32DP210bcrabl cell line at 100 nM. This suppression is non selective. Another acetylene, AC19, is relatively selective in suppressing the P210bcrabl dependent growth of the 32DP210bcrabl cell line.

Interestingly, when K1P or AC22 are combined with imatinib, the combinations are almost completely suppressive at 10 nM in concentration (a concentration at which neither drug is actively suppressive alone). This data suggests that the acetylene drugs are acting at a site in P210bcrabl that is different from imatinib. A series of furans was discovered which are exquisitely selective for P210bcrabl dependent growth at the 10 nM concentration level when used alone.

III.A. Specific Aims. The most important priority of these studies was to test if the suppressive activity of these drugs seen in vitro could be extended to in vivo growth. There were three specific aims outlined in the proposed grant application:

Specific Aim #1: In Vitro Drug Exposure to Test Suppression of In Vivo "Tumorigenic" CML Cells. This specific aim was designed to evaluate the in vitro toxicity of lead compounds on the tumorigenic cells in the 32Dp210Bcr-Abl cell line which are capable of forming subcutaneous tumor nodules in syngeneic Balb C mice.

Specific Aim #2: In Vitro Drug Exposure to Test Toxicity to Engrafting CML Cells Using Bone Marrow Purging Experiment. This specific aim was based on a model previously used in our laboratory which involves the irradiation of 2-3 month old female breeder Balb C mice with between 650-800 cGy of total body irradiation and then infusion of female mice with donor male marrow mixed with 32Dp210Bcr-Abl leukemia cells (male). The normal and leukemia cell mixture are exposed in vitro to the test drugs before infusion. This aim would test the safety and efficacy of the test drugs: AC19, AC22, A101, imatinib and a combination of imatinib and AC22.

Specific Aim #3. In Vivo Drug Administration for Toxicity and Efficacy Evaluation. This specific aim was to test the in vivo toxicity of the test drugs by administering different doses above and below the projected efficacious dose and then evaluating the therapeutic effect of the drugs on the growth of subcutaneous nodules of the 32Dp210Bcr-Abl positive leukemia cells.

Specific Aims #1 and #2 are the most important specific aims since they are designed to define the conditions under which CML cells can be exposed to the P210Bcr-Abl inhibitory drugs that will result in a suppressive effect of the in vitro drug exposure on in vivo growth following introduction into a test mouse.

III.B. Background and Rationale for Experimental Plan. All of the studies up to the time of the grant application had been conducted in the in vitro culture situation. It is clear that the growth of cells can be dramatically different when tested in vitro and in vivo. In the in vivo situation, a 3 dimensional matrix of

extracellular matrix proteins, and cell-cell interactions may protect cells from the effects of stresses which in vitro can kill cells or retard their growth.

There were two assays in the work carried out before the application was submitted for the effects of the acetylene and furan drugs on cells which carried the bcr-abl gene:

1. **Low Cell Density Proliferation Assay:** In this assay, the maximum stress was imposed on 32DP210Bcr-Abl by depositing 100 of these cells per well in a 100 microliter volume and adding another 100 microliters containing a 2X concentration of the test drug. The exposure was carried out for 15 minutes, and then 200 microliters of medium supplemented with 10% FBS was added. Then, the growth in the absence of IL3 was carried out (IL3 is necessary for the in vitro growth of the 32D cells without which the 32D cells undergo apoptosis). The 32D cells can be rescued by the P210Bcr-Abl. Exposure of the cells to the candidate drugs in the absence of serum was important since it was known that these drugs were sparingly soluble in acqueous solvents and they bound avidly to serum. The low cell density made the cells maximally vulnerable to the apoptosis inducing effects of the drugs because there were no extracellular matrix proteins like fibronectin which contain apoptosis rescue ligands for the alpha V beta.

2. **High Cell Density Proliferation Assay**: 32DP210Bcr-Abl cells or BAF-1P210Bcr-Abl cells were exposed at high cell density to the acetylenes or the furans in medium supplemented with 5% fetal bovine serum. The cell density in this assay was in the 100,000 cells/well, rather than the 100 cells/well. Thus, this assay was closer to in vivo conditions of exposure.

We decided to use the experimental plan outlined in Specific Aim #1 for the systematic analysis of the conditions of drug exposure that would lead to damage to the cell which would then result in reduction of the growth and survival of exposed cells when injected into the subcutaneous space of the test animals. It is well known that the behavior of cells in vitro is not always extended to the in vivo situation. This is due to the fact that the tumor cells in vivo are supported by both cell-cell ligand receptor interactions as well as interactions between extracellular matrix proteins and the cells which activate apoptosis rescue or survival signal transduction pathways.

Thus, the in vitro activity seen with some drugs is not always seen in vivo. We felt therefore that the most important priority was to test if the experiments were carried out with the most sensitive and broadest type of assay: exposing the cancer cells to the test drugs in the absence of serum followed by addition of serum and then injection of the drug exposed cells into the subcutaneous space. We were changing only one variable: the environment in which we would test for the survival and growth of the cancer cells following exposure. Anchorage dependent growth would be operative in previous in vitro data as well as in the current experiments which involved growth and survival of the cells in the subcutaneous space as the read out of the assay.

Once we defined conditions of drug exposure in vitro which lead to in vivo suppression of tumor growth, then two options are available: either to proceed to Specific Aim #2 (a plan identical to that studied in Specific Aim #1, except that the exposed cells are infused intravenously instead of subcutaneously), or to proceed to a series of structural modifications that would improve the bioavailability of the compounds. As discussed below, this decision was based on the outcome of the experiments in Specific Aim #1. On the basis of the results in Specific Aim #1, we decided to modify the ester side chain of the acetylenes (which is opposite to the amide group) so as to increase the efficacy of the acetylenes. In work outside of the scope of this grant, we will add a series of functional groups to the side chain of the ester group which would be designed to increase the solubility of the compounds. We would then test these drugs in the experimental paradigm of Specific Aim #1.

III.C. Summary of Experiments: First, we established the dose of Baf-1P210Bcr-Abl cells which when injected subcutaneously leads reproducibly to the growth of subcutaneous nodules. Then, we assessed the effect of in vitro exposure of the cells to the acetylene drugs under different conditions. The major variable that we studied was the percentage of ethanol added to the tissue culture medium in order to increase the solubility of the test drug K1P. We will use two different concentrations of ethanol for the solution of the drugs: 0%, and 0.1%. We used K1P for the test drug, since it has been shown to act at a site which is independent of imatinib and also, K1P is active in cell lines which are resistant to imatinib. In each experiment, there were 3 groups of mice: mice injected with cells not exposed to drug, mice injected sc with cells exposed to drug in the absence of serum, and mice injected sc with cells exposed to drug in the presence of serum.

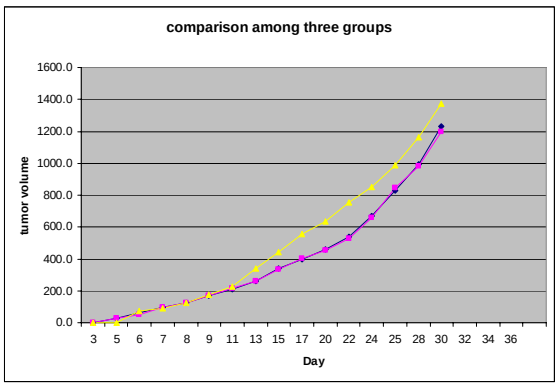
III.C.i. Dose of Baf-1P210Bcr-Abl Cells for SC Injection. 1 X 10^7 BAF-1 P210Bcr-Abl cells, when suspended in RPMI-1640 medium injected subcutaneously was found to generate reproducibly subcutaneous nodules which grow to 200 cu mm in volume in 10 days and 400 cu mm in 20 days.

III.C.ii. K1P Acetylene. Crystalline K1P which was synthesized at Yale University in the laboratory of David Austin was submitted to a contract laboratory for Mass Spectrometry and High Pressure Liquid Chromatography. The stocks of crystalline K1P which were used for these animal experiments were shown to be pure and structurally K1P.

III.D. Results.

III.D.i. First Experimental Condition for Exposure of Baf-1 Cells to K1P: Dilution in RPMI without Ethanol. In the first experimental condition, the Baf-1 cells were exposed to test drug in RPMI with 0% Ethanol. **Dilution of the drug**: the drug was dissolved in 100% ethanol and then diluted 3 times in RPMI with out ethanol. **Drug Exposure**: The cells were exposed to test drug which was dissolved in test drug in medium without ethanol for 15 minutes. The concentration used of the drug was 1 micromolar. The concentration of the drug was 1 X 10^7 cells/cc. **Experimental Groups**: There were three groups of 5 test mice each: mice that received cells which were unexposed to drug, mice that received cells exposed to the test drug without serum, and mice that were injected with cells exposed to drug diluted in medium with 5% fetal bovine serum. **Results**: The results are shown in Figure 1 below. As shown below in Figure 1, there are no differences among the 3 test groups. Thus, exposure of the Baf-1 cells to drug suspended in RPMI-1640 which is not supplemented with ethanol to solubilize the drug is without suppressive effect on the growth of the Baf-1P210Bcr-Abl cells in vivo.

Figure 1



III.D.ii. Second Experimental Condition for Exposure of Baf-1 Cells to K1P: Dilution in RPMI which is 0.1% in Ethanol and Cell Exposure to Drug in 0% Ethanol. In the second experimental condition, the Baf-1 cells were diluted in RPMI supplemented with 0.1% ethanol and then the cells were exposed to test drug in RPMI with 0% Ethanol. **Dilution of the drug**: the drug was dissolved in 100% ethanol and then diluted 3 times in RPMI which is 0.1% in ethanol. **Drug Exposure**: The cells were exposed to test drug which was dissolved in RPMI medium without ethanol for 15 minutes. The concentration used of the drug was 1 micromolar. The concentration of the drug was 1 X 10^7 cells/cc. **Experimental Groups**: There were three groups of 5 test mice each: mice that received cells which

were unexposed to drug, mice that received cells exposed to the test drug without serum, and mice that were injected with cells exposed to drug diluted in medium with 5% fetal bovine serum. **Results**: The results are shown in Figure 2 below. As shown below in Figure 2, there are no differences among the 3 test groups. Thus, exposure of the Baf-1 cells to drug diluted in 0.1% ethanol but in which the cells are exposed to drug suspended in RPMI-1640 which is not supplemented with ethanol to solubilize the drug is without suppressive effect on the growth of the Baf-1P210Bcr-Abl cells in vivo.

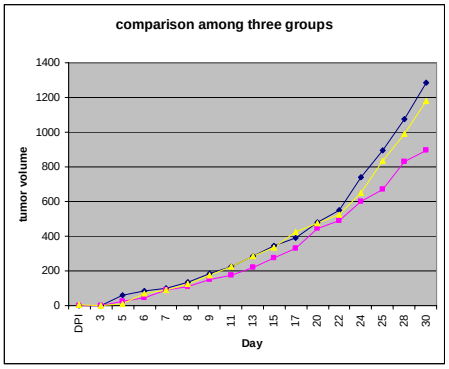


Figure 2

III.D.iii. Third Experimental Condition for Exposure of Baf-1 Cells to K1P: Dilution in RPMI which is 0.1% in Ethanol and Cell Exposure to Drug in 0.1% Ethanol. In the third experimental condition, the Baf-1 cells were diluted in RPMI supplemented with 0.1% ethanol and exposed to test drug in RPMI with 0.1% Ethanol. **Dilution of the drug**: the drug was dissolved in 100% ethanol and then diluted 3 times in RPMI supplemented with 0.1% ethanol. **Drug Exposure**: The cells were exposed to test drug which was dissolved in test drug in RPMI medium which is 0.1% ethanol for 15 minutes. The concentration used of the drug was 1 micromolar. The concentration of the drug was 1 X 10^{7} cells/cc. **Experimental Groups**: There were three groups of 5 test mice each: mice that received cells which were unexposed to drug, mice that received cells exposed to the test drug without serum, and mice that were injected with cells exposed to drug diluted in medium with 5% fetal bovine serum. **Results**: The results are shown in Figure 3 below. As shown below in Figure 3, the cells which were exposed to K1P drug which was diluted in 0.1% ethanol and also with which the cell exposure was carried out in 0.1% ethanol (see line defined by the red squares and the red line) grew more slowly than the cells not exposed to drug or the cells exposed to drug in the presence of serum (blue diamonds and yellow triangles). Thus, exposure of the Baf-1 cells to drug diluted in 0.1% ethanol but in which the cells are exposed to drug suspended in RPMI-1640 which is 0.1% ethanol to solubilize the drug generates a statistically significant delay in the in vivo growth of the tumor cells. Thus, K1P when diluted and dissolved in 0.1% ethanol damages the Baf-1P210Bcr-Abl cells sufficiently that the growth of the Baf-1P210Bcr-Abl cells in vivo is suppressed.

Interestingly, the addition of serum to the RPMI medium which is supplemented with 0.1% ethanol blocks the suppressive effect of the K1P on the Baf-1 cells. This suggests that the drug needs to be engineered on the ester side chain to improve its bioavailability to cells in the presence of extracellular proteins like serum.

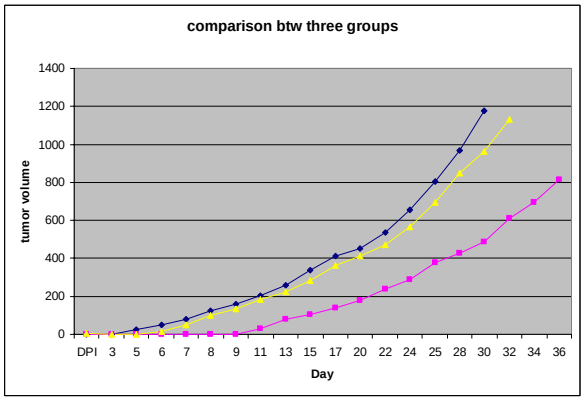


Figure 3

III.E. Toxicity Analysis: Observation of the mice injected sc with cells exposed to K1P drug dissolved in 0.1% ethanol did not reveal any toxicity: no sudden deaths, no decrease in activity, no hunching, no ruffled fur. Thus, the subcutaneous administration of cells which have been exposed to K1P in a 0.1% ethanol RPMI vehicle in doses which suppress the in vivo growth of the Baf-1P210Bcr-Abl cells does not generate toxicity.

III.F. Summary Conclusions. Baf-1P210Bcr-Abl cells which were exposed to K1P in the absence of IL3 and in medium supplemented with 0.l% ethanol at 1 micromolar can suppress the growth of the Baf-1P210Bcr-Abl

cells. It is clear on the basis of these results that further modification of these drugs so as to increase their solubility in acqueous solvents, and to increase their bioavailability to cells in the presence of serum will be necessary before further experimentation in animals can be carried out.

IV. KEY RESEARCH ACCOMPLISHMENTS.

The following was accomplished in the funding period of one year:

- Establishment of a model for in vivo testing of the acetylene K1P.
- Identification of a concentration of the K1P drug which was sufficient to damage the Baf-1P210Bcr-Abl cells sufficiently so that the in vivo growth of the cells would be delayed.
- Systematic analysis of the dilution and exposure conditions which lead to sufficient biological activity of the drug to damage the Baf-1P210Bcr-Abl cells under conditions in which they are dependent on P210Bcr-Abl to suppress the in vivo growth of the Baf-1P210Bcr-Abl cells.
- Establishment of the fact that the presence of protein in the medium used for exposure of cells to K1P blocks the activity of the drug.
- Establishment of the requirement for structure/activity studies with the acetylenes in a series of compounds in which the chemical functionalities which could increase solubility and bioavailability of the drug in the presence of serum is a requirement for going forward with additional animal studies.

V. REPORTABLE OUTCOMES.

The following reportable outcomes resulted from this work:

A. A paper has been published on this work in Molecular Cancer Therapeutics. (Lerma, et al., see appendix).
B. Demonstration that K1P is active sufficiently to suppress the growth of Baf-1P210Bcr-Abl cells when they are injected subcutaneously following a 15 minute exposure.
C. Demonstration that the K1P acetylene needs further structural modification to make it sufficiently bioavailable when the cells are exposed to K1P in the presence of serum
D. Plan for structural modification for the acetylenes going forward.

VI. CONCLUSIONS.

These experiments show that the activity of the acetylenes is sufficiently strong to damage cells which are dependent on P210Bcr-Abl for their survival so that their in vivo growth is suppressed.

These experiments have shown that these drugs require 0.1% ethanol in the medium to generate sufficient solubility for biological activity in vivo.

These experiments have shown that there is a need to structurally modify the ester side chain so that the bioavailability to cells in the presence of serum is increased.

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Paid Personnel:

The following persons were paid from this grant.

Albert B. Deisseroth, Principal Investigator
Jonathan Maynard, Research Technician

VIII. APPENDICES.

A. Paper Published from work supported in part by DOD CML Grant:

Lerma E, Nguyen AV, Wang T, Twilling A, Melo J, Austin D, and Deisseroth A. Use of combinatorial structural variation of top design new drugs for CML. Mol Cancer Ther, 6: 655-666, 2007.

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Novel compounds with antiproliferative activity against imatinib-resistant cell lines

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Abstract

Chronic myelogenous leukemia is caused by the *Bcr-Abl* hybrid gene that encodes the p210Bcr-Abl chimeric oncoprotein. Although it reduces the total body burden of leukemia cells, the use of imatinib mesylate as a single agent may be accompanied by the evolution of resistance due mainly to the acquisition of point mutations. Imatinib has been combined with drugs that inhibit both the active and the inactive states of the p210Bcr-Abl kinase. These combinations have reduced but not completely eliminated the rate at which point mutations are acquired in the p210Bcr-Abl kinase. Thus, it is important to identify additional new inhibitors of the p210Bcr-Abl kinase. One possible method to prevent evolution of resistance is to simultaneously use multiple kinase inhibitors each with a different mechanism of action. To identify such a new class of inhibitors that could suppress the growth of chronic myelogenous leukemia cells and prevent the evolution of cells that are resistant to imatinib, we screened two low-complexity libraries of compounds based on planar and linear scaffolds. These libraries were screened using a cell-based assay for molecules that suppress p210Bcr-Abl–dependent cell growth. The application of this method resulted in the isolation of two new classes of drugs, both of which inhibited imatinib-resistant cells in the low micromolar range. Some of these drugs were potent inhibitors not only of Abl tyrosine kinase but also of the Src, Lyn, and Fyn tyrosine kinases. [Mol Cancer Ther 2007;6(2):OF1–12]

Introduction

Chronic myelogenous leukemia (CML) is caused by the t(9;22) reciprocal chromosomal translocation that results in the formation of *Bcr-Abl* gene. The protein product of this chimeric gene is the tyrosine kinase p210Bcr-Abl fusion protein that is responsible for the clinical phenotype of CML. This oncoprotein kinase is constitutively active and is located in the cytoplasm. The augmented tyrosine kinase activity of p210Bcr-Abl produces phosphorylation of many downstream substrates.

In the absence of therapy, the genetic instability conferred on CML cells by the p210Bcr-Abl kinase leads to continual evolution of the phenotype of the CML cells from an indolent disorder associated with an elevated circulating myeloid cell mass to a fulminant acute leukemic disorder in which patients die of bleeding and infection (1). Allogeneic bone marrow transplantation was the first curative therapy for CML, which resulted in an overall survival of 50% to 80% of the patients so treated (2). Offsetting this benefit was the transplant-related mortality from acute or chronic complications. IFN resulted in cytogenetic remissions in 20% of low Sokal index patients but at a considerable cost in the quality of life for those individuals so treated (3).

Imatinib, a specific inhibitor of the *Bcr-Abl* tyrosine protein kinase, has revolutionized the therapy of CML because it selectively suppresses the number of leukemia cells without significant toxicity to normal hematopoietic cells (4–6). Offsetting this enormous benefit is the evolution of resistance in chronic- and advanced-phase patients (7, 8). Overexpression of p210Bcr-Abl protein, overexpression of multidrug resistance 1 P-glycoprotein, overexpression of other kinases, such as the Src-related kinases Lyn and Hck, and acquisition of point mutations in the *Bcr-Abl* gene have been shown to contribute to the evolution of clinical resistance to imatinib (7–9). Like the p210Bcr-Abl kinase, Lyn kinase is involved in the response of hematopoietic cells to exposure to DNA-damaging agents, such as ionizing radiation (10).

Several families of compounds have been reported to suppress CML cells following the first reports of imatinib activity (4–11). We investigated synthetic variations of chemical functionalities based on linear or planar scaffolds to identify new compounds that inhibited the p210Bcr-Abl–dependent proliferation of cell lines at low nanomolar concentrations in a highly selective fashion and that suppressed the growth of imatinib-resistant cells *in vitro*

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(12, 13). Two types of scaffold molecules were evaluated in this study, acetylenes, representing a linear scaffold, and furans, representing a planar scaffold, from which chemical functionalities could be projected in a diversity-based approach. In addition, the acetylenes are also precursors of the furan scaffold molecules, which facilitated a 2-fold investigation of the molecules from our combinatorial synthetic strategy (12–14). The results reported in this article show that both the furans and the acetylene scaffolds can be used to generate highly selective inhibitors of CML cells, some of which suppress the growth of imatinib-resistant cell lines.

Materials and Methods

Reagents and Compounds

Murine interleukin-3 (IL-3) culture supplement was provided by BD Biosciences (Bedford, MA). [γ-^{32}P]ATP was obtained from ICN (MP Biomedicals, Salon, OH). Src(p60c-src) and Lyn(p56) partially purified recombinant enzymes and their corresponding buffers and substrates were obtained from Upstate Biotechnology (Lake Placid, NY). Recombinant v-Abl protein tyrosine kinase and reaction buffer were purchased from Calbiochem (EMD Biosciences, San Diego, CA) and New England Biolabs (Ipswich, MA). In the first year of the research, the synthetic Abl peptide substrate EAIYAAPFAAKKK was synthesized at the Yale Keck facility from the sequence provided by Calbiochem. In later years, this peptide was purchased from New England Biolabs. Imatinib mesylate (Gleevec) is commercially available from Novartis (Cambridge, MA).

The acetylenes and furans were synthesized in the laboratory of David J. Austin in the Department of Chemistry at Yale University (New Haven, CT). All compounds underwent high-pressure liquid chromatography purification and mass spectrometry to determine the structural integrity of each compound both at the time of isolation and in a time frame that was proximate to the biological analysis. The acetylenes were created in one step by the coupling of a propiolate anion and isocyanate. These acetylenes were then used as a precursor in the synthesis of the furans.

The furan synthesis was based on early research in the Austin laboratory, which was centered on the concept of adapting the 1,3-dipolar cycloaddition reaction between a diazo ketone and assymmetrically substituted acetylenes using a rhodium(II)-mediated catalysis (13). This synthetic path was part of a larger strategy to create a planar system for presentation of a wide variety of functional groups for molecular recognition. Previous work in the Austin laboratory had suggested that a priority for inhibitory activity of the Bcr-Abl kinase was to clear the region of space on the amide side of the furan so that the molecule could interact with the Bcr-Abl kinase pocket. Although this interaction of these compounds with the kinase was conjecture, the Austin laboratory data on the furans suggested that the absence of steric hindrance on the amide side of the furan would increase the inhibition of the p210Bcr-Abl–dependent growth of cell lines in the low- and high-density proliferation assays.

Cell Lines

Generation of the 32Dtetp210Bcr-Abl Cell Line. The 32D cell line was initially obtained from Joel Greenberg at the University of Pittsburgh School of Medicine (Pittsburgh, PA). The introduction of a 'Tet-off' tetracycline-inducible *Bcr-Abl* transcription unit into the 32D myeloid cell line (which is IL-3 dependent for growth *in vitro*) generated a cell line that was IL-3 independent when Bcr-Abl was expressed in the absence of tetracycline. This cell line was designated 32Dtetp210Bcr-Abl (15, 16).

Imatinib-Resistant Cell Lines. The imatinib mesylate–resistant cell lines Baf/BCR-ABL-r and LAMA84-r and their imatinib-sensitive parental cell lines Baf/BCR-ABL-s and LAMA84-s were derived as described previously (17, 18).

Proliferation Assays

Low-Density Cell Proliferation Assay of 32Dtetp210Bcr-Abl and 32D Cell Lines in the Presence and Absence of Test Drugs. Between 50 and 100 32D and 32Dtetp210Bcr-Abl cells in medium without serum were exposed to the test compound for 15 min. An equal volume of 20% serum-supplemented tissue culture medium was then added, and the cells were inoculated in 32 wells of a 96-well plate for a total final volume of 100 μL/well. The end point of the assay was the percentage of the 32 wells filled with live cells after 7 days of incubation at 37°C.

During the growth assays, 32D cells were cultured in the presence of IL-3 and in the absence of tetracycline, whereas the 32Dtetp210Bcr-Abl cell line was grown in the absence of both tetracycline and IL-3. Each experiment was done once. However, a total of 32 wells was monitored for cell growth for each drug. Because each well contained between 50 to 100 cells, the addition of a drug totally suppressed all cell growth or a pellet developed in a well filled with viable cells. The formation of the pellet was the end point of the assay.

If a compound suppressed the growth of 32Dtetp210Bcr-Abl cell line without affecting the growth of the parental 32D cell line, then the compound was considered to be selectively inhibitory for p210Bcr-Abl–dependent growth. Compounds that would be bound to serum proteins due to low solubility in aqueous solvents could be studied because they were solubilized in 0.1% ethanol and added to the cells initially in the absence of serum. Finally, we selected the end point of inhibition of p210Bcr-Abl–dependent proliferation of 32Dtetp210Bcr-Abl cells to be able to identify inhibitors of the p210Bcr-Abl kinase as well as inhibitors of its downstream targets on which the continued expansion of the 32Dtetp210Bcr-Abl cells depends.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium High-Density Cell Proliferation Assay. Cell proliferation assays of the imatinib-sensitive and imatinib-resistant cell lines were done in triplicate using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; CellTiter 96 Aqueous, Promega, Madison, WI), which measures the numbers of viable cells. Between 2×10^3 and 2×10^4 imatinib-resistant cells were washed twice in RF-10 and plated in quadruplicate in the wells of a 96-well

microtiter plate in 100 μL of RF-10 medium supplemented with various doses of test compounds. Controls using the same concentration of imatinib without cells were set up in parallel. The plate was then incubated for 72 h at 37°C. Two hours after MTS addition, the plate was read in a microplate autoreader at a wavelength of 490 nm. Results were expressed as the mean absorbance of the four-well set of each compound dose. All experiments were repeated at least thrice.

Growth Assay Conducted by the National Cancer Institute of the NIH. A panel of human tumor cell lines was screened by the NIH for suppression by AC22 and AC19. A 96-well format was used with cell densities ranging from 5,000 to 40,000 per well. The test drugs were dissolved in DMSO at the stated concentrations and added to the cell cultures. Afterwards, the cell growth was measured by absorbance measurements.

Gel Electrophoresis Assay of Kinase Activity

Compounds identified in the cell density proliferation assays were also tested for inhibition of the v-Abl and Src protein kinases in cell-free in-gel kinase assays. These assays have been carried out by phosphorylating a peptide substrate with [γ-^{32}P]ATP followed by separation of the ^{32}P-peptide product from the unreacted [γ-^{32}P]ATP. The kinase reaction mixture contained the given enzyme with the addition of testing compounds at varying concentrations. After incubation in enzyme buffer for 2 h at 0°C on ice, [γ-^{32}P]ATP, ATP, the protein or peptide substrate were added. The reaction mixture was then incubated at 30°C for 10 min at a final volume of 25 μL/sample. The reaction was terminated by the addition of Tris-tricine sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 min in tightly capped tubes. The reaction products were separated by electrophoresis on Tris-tricine 16.5% gel (Bio-Rad), and the ^{32}P-labeled protein or peptides were visualized by autoradiography. Each assay contained an untreated control, in which a volume of diethylpyrocarbonate-treated water was added to replace the volume of the compound added in the treated samples.

Cell-Free Kinase Inhibition Assay

A 96-well plate assay has been carried out with the v-Abl–dependent kinase colorimetric reaction. Plates were precoated with a random polymer substrate containing multiple tyrosine residues, which may be phosphorylated by protein tyrosine kinases in the sample being assayed. The detector antibody is a purified horseradish peroxidase–conjugated, mouse monoclonal antibody that recognizes phosphotyrosyl residues. The v-Abl kinase inhibitory activity of a compound was detected by a decrease in the intensity of the colorimetric reaction. The results were assayed by a plate reader. The compound was added in triplicate, and concentration curves for each compound were carried out at four concentrations: 10 μmol/L, 1 μmol/L, 100 nmol/L, and 10 nmol/L. The analysis of the untreated control samples was carried out with the addition of diethylpyrocarbonate-treated water. The negative controls were studied without substrate and without compounds and without kinase and without compounds.

Cell-Free Inhibition of a Kinase Panel by K1P and AC22

A panel of 60 kinases (Upstate Biotechnology) was studied for cell-free inhibition to characterize the targets of the acetylene compounds K1P and AC22.

Results

Synthesis of Acetylenes and Furans

Our initial hypothesis was that a planar molecule, such as a furan, could inhibit the p210Bcr-Abl tyrosine-specific protein kinase. This was based on the fact that tyrosine kinases are susceptible to inhibition in the ATP pocket by heterocyclic molecules that possess a flat architecture that can reach within the ATP pocket yet do not disturb the conformational change in the kinase domain as it closes down on the binding molecule, in this case our inhibitor. By flanking this flat, planar scaffold with a wide range of chemical species, we endeavored to discover a p210Bcr-Abl kinase inhibitory molecule that was predicted to contain one or more monomeric functional units.

Early research centered on the concept of adapting the 1,3-dipolar cycloaddition reaction to combinatorial chemistry to create diverse libraries of molecules that could be screened for biological activity (12–14). The initial hypothesis was that the 1,3-dipolar cycloaddition of diazo ketones with activated acetylenes followed by heat induced cycloreversion (see Fig. 1A for this reaction) yielded a planar molecule known as a furan. This could serve as the core of a molecular scaffold from which a diverse array of chemical functionalities could be displayed, thereby generating a low-complexity library of chemicals to be tested as inhibitors of the 32Dtetp210Bcr-Abl cell line. We intended to screen both the acetylene precursors and the furan compounds for their ability to inhibit the 32Dtetp210Bcr-Abl cell line. We therefore synthesized a series of furan molecules from a series of acetylene precursor compounds.

The current lead acetylene molecules, K1P, AC19, and AC22, were selected from a study involving the acetylenic structures shown in Fig. 1B and represent a diverse array of chemical functionality and scaffold manipulation. The inhibitory activity of each chemical for the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay is shown in Table 1.

Relationship of Size of Functional Groups Adjacent to the Acetylenic Ester and Growth Inhibition of the 32Dtetp210Bcr-Abl Cells in the Low-Density Cell Proliferation Assay

Testing of a series of acetylenic scaffolds with different substituent chemical functionalities allowed us to determine the best positions on the linear acetylene scaffold for growth inhibition of the 32Dtetp210Bcr-Abl cell line. As shown in Fig. 2A, both K1P (Fig. 2A, *1*) and AC1 (Fig. 2A, *2*) inhibited the growth of the 32Dtetp210Bcr-Abl cell line to similar degrees in the low-density cell assay despite the difference in the size of the alkyl groups on their ester moieties. K1P contains a methyl ester, whereas AC1 contains a tertiary butyl ester. This suggested that the size of the alkyl groups on the ester portion of the acetylenes

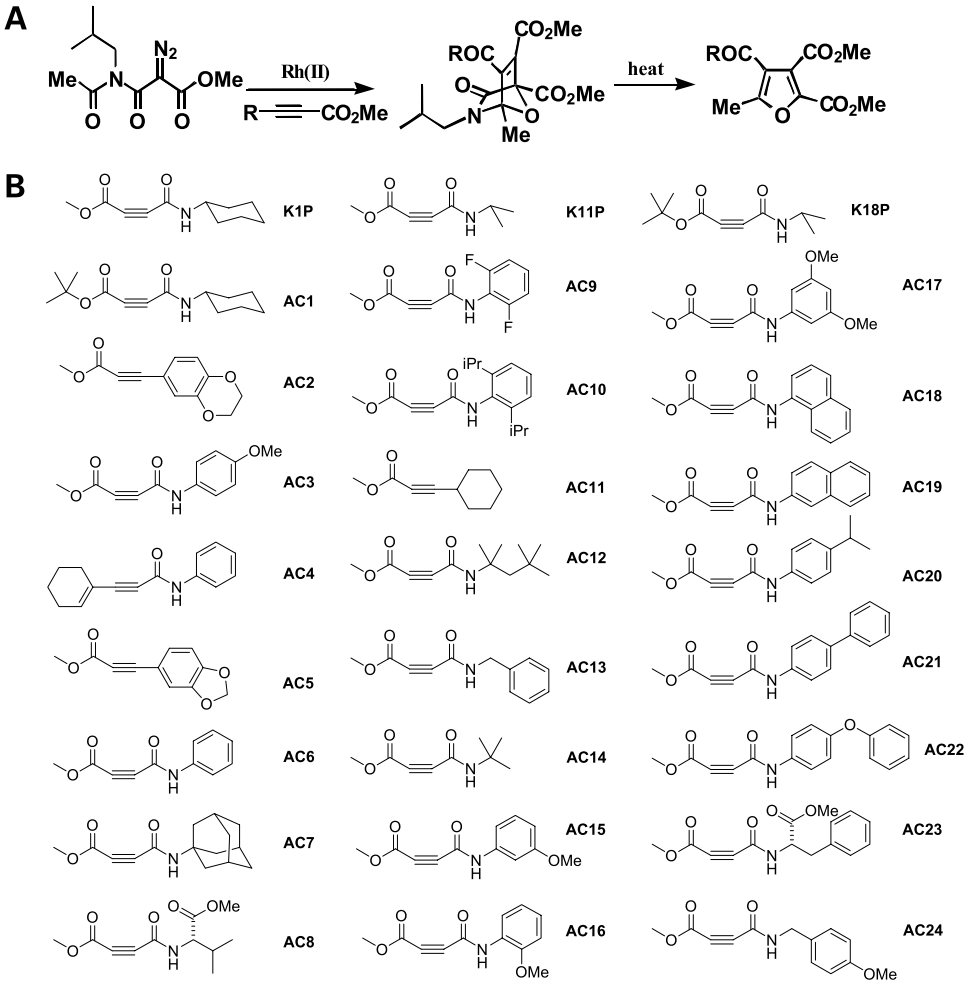

Figure 1. A, cycloaddition reactions for synthesis of furans from acetylenes. A rhodium catalyst is used to generate the furan planar scaffold from the acetylene linear scaffold. **B,** library of acetylenes. These compounds were manually synthesized for the analysis of the relationship between substitution of the various sites on the acetylenes and the suppression of p210Bcr-Abl–dependent growth.

was not a crucial part of the scaffold in terms of suppressing the growth of the 32Dtetp210Bcr-Abl cell line. AC1 was much more selectively inhibitory for p210Bcr-Abl–dependent growth than was K1P.

Relationship of Size of Groups on the Acetylenic Amide and Growth Inhibition of 32Dtetp210Bcr-Abl Cells in the Low-Density Cell Proliferation Assay

K1P is inhibitory for the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay, whereas K11P is not (see Table 1). K1P has a cyclohexane on the amine, whereas K11P has a less bulky group at that site (see Fig. 1B). These data suggest that the size of the alkyl groups on the amide may affect the level of inhibitory activity of the acetylenes for the growth of the 32Dtetp210Bcr-Abl cell line.

Importance of the Acetylenic Amide or the Ester Groups for Inhibition of the Growth of the 32Dtetp210 Bcr-Abl Cell Line in the Low-Density Cell Proliferation Assay

We next evaluated the importance of the acetylenic ester or amide groups for growth suppression of the 32Dtetp210Bcr-Abl cell line. K1P (see Fig. 2B, *1*) suppressed 32Dtetp210Bcr-Abl cell growth, whereas AC4 (see Fig. 2B, *2*), which lacks an ester but contains a similar amide group, did not. Conversely, AC2, which contains the ester group but not the amide, was found to be suppressive of the growth of Bcr-Abl–expressing cells in the same assay. Therefore, we can conclude that the ester carbonyl is essential for suppressing the growth of the 32Dtetp210Bcr-Abl cell line. As described in the preceding section, the size of the acetylenic ester substituent itself is less important. In contrast, the size of the group on the acetylenic amide is very important for a compound to inhibit the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay.

Inhibitory Effect of the Acetylenic Compounds K1P, AC19, and AC22 on the Growth of the 32Dtetp210Bcr-Abl Cell Line in the Low-Density Cell Proliferation Assay

Based on the experimental results shown in Figs. 1B and 2A and B and Table 1, three of the acetylenes were chosen for in-depth study: K1P, AC19, and AC22. Pilot experiments (data not shown) had established that 1 μmol/L AC19 suppressed both the proliferation of the parental 32D cell line (in the presence of IL-3) and the 32Dtetp210Bcr-Abl cells (in the absence of IL-3). As shown in Fig. 2C, AC19 at

100 nmol/L is almost totally inhibitory of the growth of the 32Dtetp210Bcr-Abl cell line in the absence of IL-3. In contrast, AC19 does not suppress the growth of the 32D cell line in the presence of IL-3. Thus, AC19 is selectively inhibitory of the cell line expression of the *Bcr-Abl* gene.

As shown in Fig. 2C, AC22 at 100 nmol/L inhibited the growth not only of the 32Dtetp210Bcr-Abl cell line but also of the parental 32D cells (i.e., its toxicity was nonselective). K1P inhibits the growth of the 32Dtetp210Bcr-Abl cell line at 100 nmol/L as well, with less toxicity to the 32D cell line in the presence of IL-3. In addition, it is clear that neither the K1P nor the AC19 nor the AC22 has a significant inhibitory effect on the growth of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L concentration when either compound is used alone in the low-density cell growth inhibition assay.

Interaction of the Acetylenes and Imatinib in Suppressing the Growth of the 32Dtetp210Bcr-Abl Cell Line

We then studied the effect of AC19, AC22, and K1P when combined with imatinib on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay. As shown in Fig. 2C, the combination of AC19 and imatinib (STI571) at a concentration of 10 nmol/L of each drug inhibited the growth of the 32Dtetp210Bcr-Abl cell line by 15%, which is less than the sum of the inhibitory effects of both drugs (25% suppression) when they are used separately.

In contrast, when AC22 and imatinib were combined at a 10 nmol/L concentration of each drug (see Fig. 2C), the growth inhibition of 32Dtetp210Bcr-Abl cells (80% suppression) was greater than the sum of the growth inhibition seen when each of the drugs was used alone (25% suppression by imatinib and 10% by AC22 when used alone). A similar enhanced inhibitory effect on the growth of the 32Dtetp210Bcr-Abl cell line is seen when K1P and imatinib are used together at 10 nmol/L each: at least 45% inhibition is seen for the combination, whereas at the same individual concentration imatinib alone induces only 25% suppression of cell growth and no inhibition is observed for K1P alone.

Table 1. Inhibition of growth of 32D and 32Dtetp210Bcr-Abl by acetylenes in low-density cell proliferation assay

Compound	Tested in serum	Tested serum-free	MIC	Selectivity
AC1	+*	−†	100 nmol/L	Selective
AC2	+	−	10 nmol/L	Selective
AC3	+	+*	10 nmol/L‡	Selective
AC4	+	−	Undetectable	
AC5	−†	+	Not done	
AC6	−	+	100 nmol/L	Selective
AC7	−	+	Not done	
AC8	−	+	100 nmol/L	Selective
AC9	−	+	Not done	
AC10	−	+	100 nmol/L	Not selective
AC11	−	+	Not done	
AC12	−	+	100 nmol/L	Not selective
AC13	+	+	100 nmol/L	Not selective
AC14 (K13P)	−	−	100 nmol/L	Selective
AC15	−	+	100 nmol/L	Not selective
AC16	−	+	Not done	
AC17	−	+	1 μmol/L	Not selective
AC18	−	+	100 nmol/L	Selective
AC19	−	+	100 nmol/L	Selective
AC20	+	−	100 nmol/L	Selective
AC21	−	+	100 nmol/L	Selective
AC22	+	+	100 nmol/L§	Not selective
AC23	+	−	1 μmol/L	Not selective
AC24	−	+	10 nmol/L	Not selective
K1P	+	+	100 nmol/L‡	Selective
K11P	+	−	Undetectable	
K18P	+	−	100 nmol/L	Selective
Imatinib	−	+	100 nmol/L	Selective

NOTE: Selectivity is defined by inhibition of the 32Dtetp210Bcr-Abl cell line in the absence of IL-3, which is twice the level of inhibition seen with the 32D cell line in the presence of IL-3.
Abbreviation: MIC, minimal inhibitory concentration.
*The test was carried out without serum.
†The test was carried out with serum.
‡The minimal inhibitory concentration was carried with serum.
§The minimal inhibitory concentration was carried without serum.

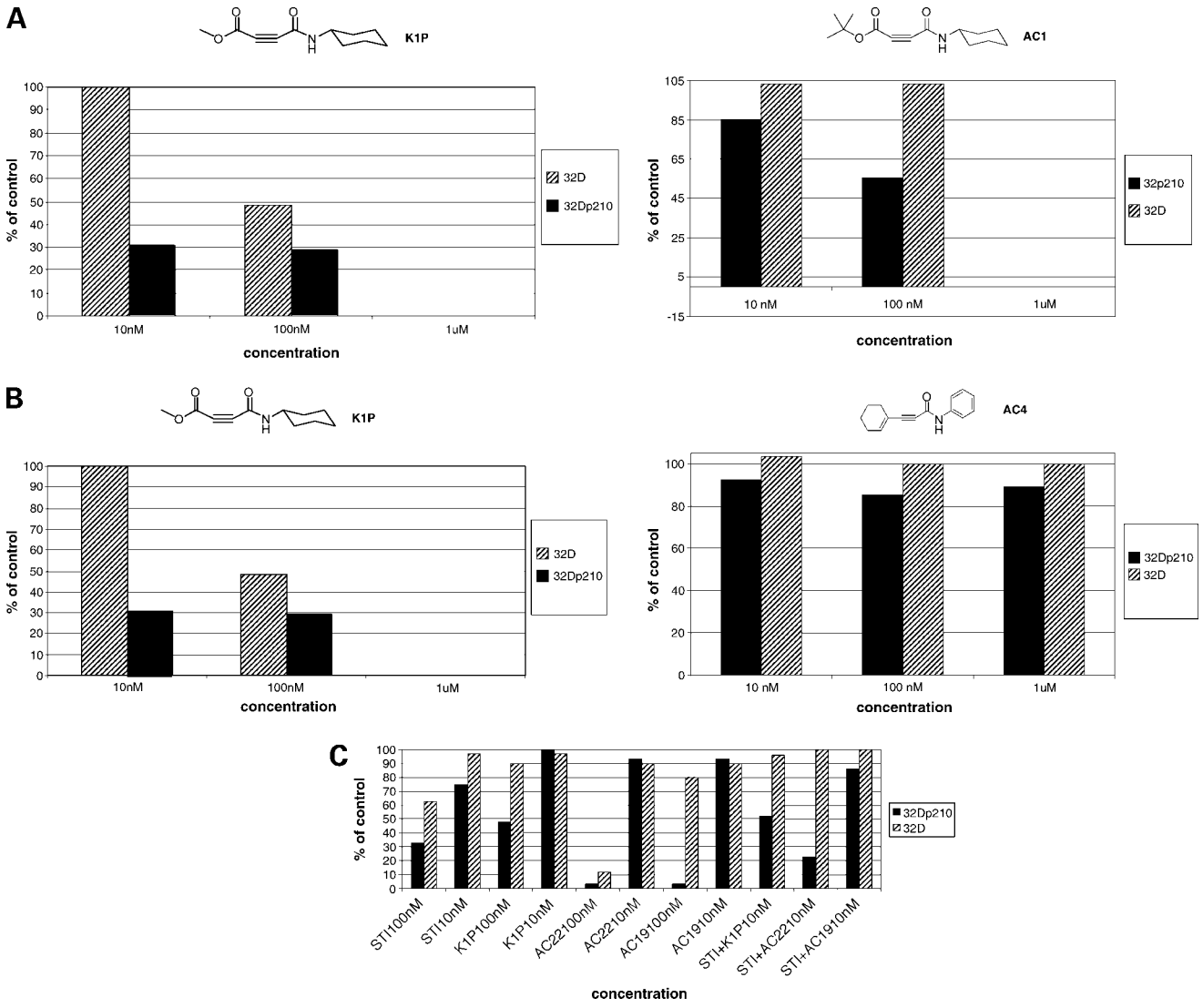

Figure 2. **A,** effect of substitutions on the inhibitory activity of the acetylenic ester on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. Comparison of the inhibitory effect of K1P (*left*) with AC1 (*right*) acetylenic compounds on the growth of the 32Dtetp210Bcr-Abl cell line was carried out in the low-density cell proliferation assay. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*. **B,** effect of substitutions on the inhibitory activity of the acetylenic amide on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. Comparison of the inhibitory effect of K1P (*left*) with AC4 (*right*) acetylenic compounds on the growth of the 32Dtetp210Bcr-Abl cell line was carried out in the low-density cell proliferation assay. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*. **C,** comparison of the suppression of growth of the 32Dtetp210Bcr-Abl cell line by the acetylenes AC19, AC22, and K1P with imatinib mesylate in the low-density cell proliferation assay. The suppressive effect of imatinib mesylate with the acetylenes AC19, AC22, and K1P on the growth of 32Dtetp201Bcr-Abl and 32D in the low-density cell assay was carried out. The acetylenes were tested alone and in combination with imatinib mesylate. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*.

Effect of K1P, AC19, and AC22 on the Growth of Imatinib-Resistant Cell Lines in the High-Density Cell Proliferation Assay (MTS)

The results with the 32Dtetp210Bcr-Abl cell line in the low-density cell assay suggested that at least some of the acetylenes (AC22 and K1P) might suppress the growth of imatinib-resistant cells. Therefore, we added each of the acetylene drugs (AC19, AC22, and K1P) to cultures (one drug per well) of the Baf/*BCR-ABL*-r and LAMA84-r cell lines, which are resistant to 1 μmol/L imatinib. These cell lines (17, 18) are grown continuously in 1 μmol/L imatinib to maintain the imatinib-resistant phenotype. Parental imatinib-sensitive variants of these cell lines (Baf/*BCR-ABL*-s and LAMA84-s) were maintained in parallel cultures without imatinib to be used as controls.

As shown in Fig. 3 and Table 2, at 1 μmol/L, K1P, AC22, and AC19, respectively, suppress 45%, 65%, and 40% of the growth of the Baf/*BCR-ABL*-r cell line in which the

mechanism of drug resistance is overexpression of *Bcr-Abl* gene. The ratio of the growth of the Baf/*BCR-ABL*-r cell line (see Fig. 3A) to that of Baf/*BCR-ABL*-s cell line (see Fig. 3B) in the presence of 1 μmol/L imatinib was 2.4. In contrast, the ratios of the growth of the Baf/*BCR-ABL*-r cell line (Fig. 3A) to that of the Baf/*BCR-ABL*-s line (Fig. 3B) in the presence of 1 μmol/L of K1P, AC19, and AC22 were 1.2, 2.2, and 1.3, respectively. This suggested that the level of resistance of the Baf/*BCR-ABL*-r cell line to the K1P, AC19, and AC22 acetylenic drugs was less than to imatinib.

When the inhibitory effect of each acetylenic compound was compared with imatinib at 1 μmol/L, the ratios of the growth of Baf/*BCR-ABL*-r cells in the presence of imatinib divided by their growth in the presence of K1P, AC19, or AC22 were 1.8, 1.5, and 2.5, respectively. This suggests that the acetylenic compounds are more inhibitory to the Baf/*BCR-ABL*-r cell line than is imatinib.

We also tested the effect of K1P, AC19, and AC22 on the growth of the LAMA84-s imatinib-sensitive cell line (see Fig. 3F and Table 2) and the LAMA84-r imatinib-resistant cell line (see Fig. 3E and Table 2), in which the mechanisms of imatinib resistance are overexpression of *Bcr-Abl* and *multidrug resistance 1* genes. The ratio of the growth of the LAMA84-r cell line (Fig. 3E) to that of the LAMA84-s cell line (Fig. 3F) in the presence of 1 μmol/L imatinib was 4.1. In contrast, the ratios of the growth of the LAMA84-r cell line to that of LAMA84-s in the presence of 1 μmol/L of K1P, AC19, and AC22 were 2.8, 2.8, and 2.7, respectively. Although these data suggest that the

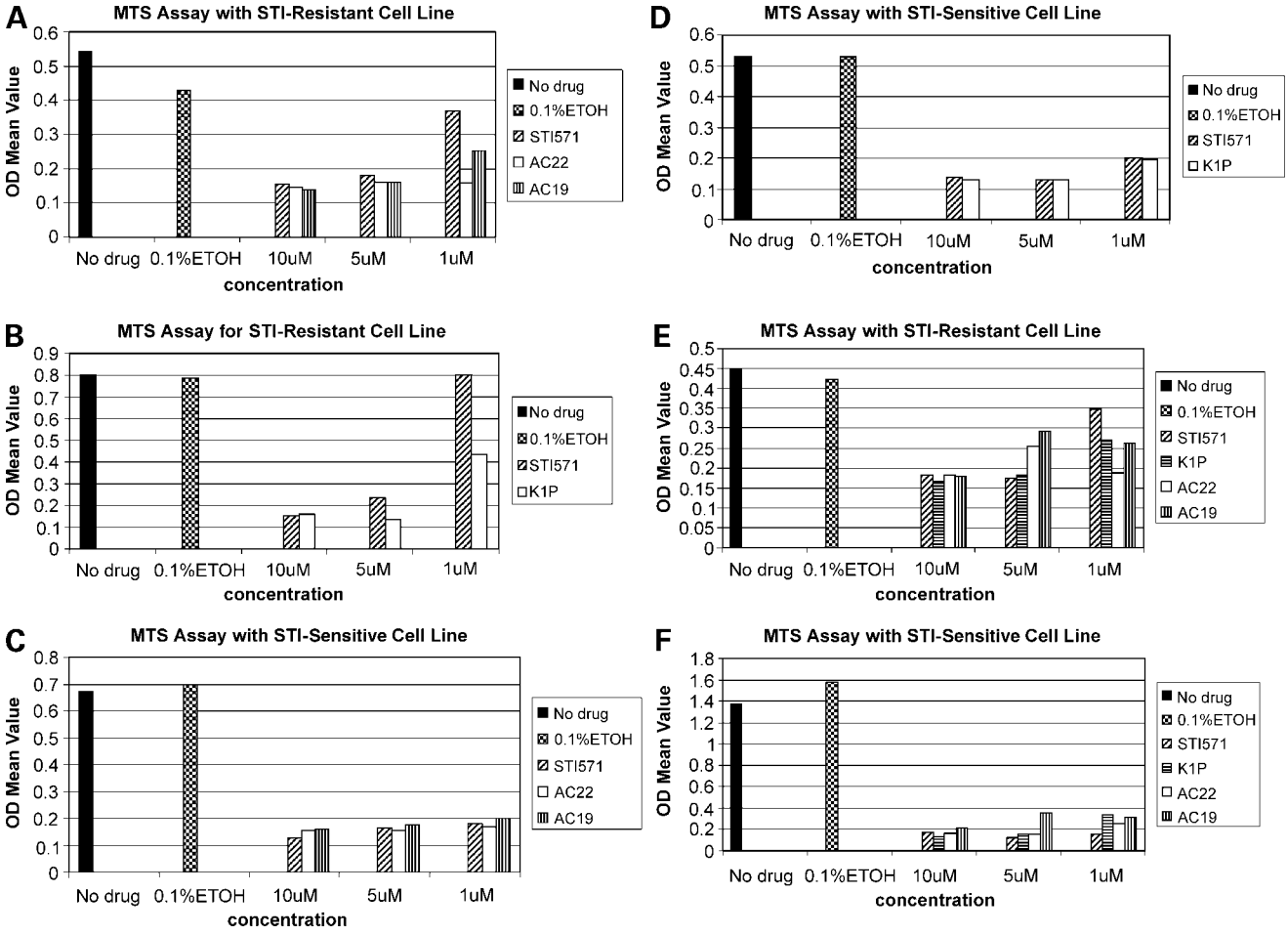

Figure 3. Effect of K1P, AC19, and AC22 acetylenes on the growth of imatinib-resistant and imatinib-sensitive cell lines as assessed by the high-density MTS cell proliferation assay. **A,** Baf/*BCR-ABL*-r comparison of the inhibitory effect of AC19 and AC22 with STI571 (imatinib) on Baf/*BCR-ABL*-r. **B,** comparison of the inhibitory effect of K1P with STI571 (imatinib) on Baf/*BCR-ABL*-r. **C,** comparison of the inhibitory effect of AC19 and AC22 with STI571 (imatinib) on Baf/*BCR-ABL*-s. **D,** comparison of the inhibitory effect of K1P with STI571 (imatinib) on Baf/*BCR-ABL*-s. **E,** comparison of the inhibitory effect of K1P, AC19, and AC22 with STI571 (imatinib) on LAMA84-r. **F,** comparison of the inhibitory effect of K1P, AC19, and AC22 with STI571 (imatinib) on LAMA84-s. The growth of the Baf/*BCR-ABL*-r cells in 1 μmol/L STI571 (imatinib) was significantly greater than the growth in 1 μmol/L of AC19 and AC22 at the $P < 0.05$ level. The growth of the Baf/*BCR-ABL*-r cells in 1 μmol/L AC19 was significantly greater than in 1 μmol/L AC22 at the $P < 0.05$ level. The growth of the LAMA84-r cells in 1 μmol/L STI571 (imatinib) was greater than the growth in 1 μmol/L AC22 at the $P < 0.05$ level. Analysis was not conducted for the significance of differences at >1 μmol/L concentration.

Table 2. Effect of AC19, AC22, and K1P compared with STI571 (imatinib) on the growth of Baf/BCR-ABL-r and LAMA84-r

	Absorbance from MTS assay			Absorbance from MTS assay		
	Baf/BCR-ABL-r (Fig. 3A)			Baf/BCR-ABL-s (Fig. 3C)		
	10 μmol/L	5 μmol/L	1 μmol/L	10 μmol/L	5 μmol/L	1 μmol/L
AC19	0.138	0.163	0.270	0.155	0.179	0.157
	0.119	0.167	0.264	0.171	0.182	0.191
	0.145	0.166	0.255	0.162	0.183	0.212
	0.156	0.149	0.217	0.142	0.158	0.235
Mean AC19	0.140	0.163	0.252	0.158	0.176	0.199
AC22	0.142	0.163	0.159	0.145	0.141	0.163
	0.155	0.162	0.154	0.163	0.148	0.183
	0.144	0.161	0.158	0.158	0.157	0.153
	0.141	0.157	0.159	0.147	0.170	0.153
Mean AC22	0.146	0.161	0.158	0.153	0.154	0.166
STI571	0.164	0.196	0.398	0.121	0.169	0.199
	0.163	0.183	0.398	0.127	0.170	0.191
	0.168	0.183	0.411	0.129	0.164	0.186
	0.122	0.155	0.296	0.136	0.155	0.146
Mean STI571	0.154	0.179	0.389	0.128	0.165	0.181
	LAMA84-r (Fig. 3E)			LAMA84-s (Fig. 3F)		
	10 μmol/L	5 μmol/L	1 μmol/L	10 μmol/L	5 μmol/L	1 μmol/L
AC19	0.194	0.273	0.275	0.186	0.344	0.424
	0.184	0.343	0.256	0.227	0.368	0.296
	0.190	0.311	0.266	0.230	0.350	0.261
	0.147	0.241	0.252	0.213	0.357	0.318
Mean AC19	0.179	0.292	0.262	0.214	0.357	0.318
AC22	0.222	0.279	0.136	0.161	0.170	0.234
	0.218	0.265	0.212	0.159	0.151	0.284
	0.189	0.251	0.195	0.176	0.154	0.258
	0.100	0.218	0.207	0.177	0.157	0.263
Mean AC22	0.182	0.253	0.187	0.168	0.158	0.260
K1P	0.156	0.192	0.197	0.144	0.157	0.291
	0.168	0.208	0.302	0.117	0.158	0.416
	0.173	0.191	0.282	0.126	0.157	0.344
	0.166	0.137	0.300	0.152	0.136	0.291
Mean K1P	0.166	0.182	0.270	0.135	0.152	0.335
STI571	0.205	0.162	0.396	0.203	0.126	0.155
	0.205	0.176	0.389	0.245	0.127	0.157
	0.211	0.175	0.383	0.167	0.132	0.159
	0.106	0.179	0.219	0.093	0.089	0.140
Mean STI571	0.182	0.173	0.347	0.177	0.118	0.153

acetylenes are more suppressive of the growth of the imatinib-resistant LAMA84-r cell line than is imatinib, it is clear that the level of cross-resistance to the acetylenes in the LAMA84-r cell line is greater than that of the Baf/*BCR-ABL*-r line (see above ratios of 1.2, 2.2, and 1.3 for the Baf/BCR-ABL-r/Baf/BCR-ABL-s cell lines compared with the ratios of 2.8, 2.8, and 2.7 for the LAMA84-r/LAMA84-s cell lines).

When the inhibitory effect of the acetylenes at 1 μmol/L on the growth of the LAMA84-r cell line is compared with the growth-suppressive effect of imatinib at 1 μmol/L (as calculated by the ratio of the growth of the LAMA84-r cell line in 1 μmol/L imatinib/growth in each of the acetylenes), the K1P, AC19, and AC22 were 1.3, 1.2, and 1.4 times as potent, respectively, as imatinib. This result suggests that the suppressive effect of the acetylenic drugs in the Baf/BCR-ABL-r line (in which the ratios of the growth in imatinib/growth in the acetylene compounds K1P, AC19, and AC22 were 1.8, 1.5, and 2.5, respectively) was greater than in the LAMA84-r cell line (in which the ratios of compounds K1P, AC19, and AC22 were 1.3, 1.2, and 1.4, respectively).

Discovery of Compounds Based on the Furan Scaffold, Which Are Selectively Suppressive of the Growth of 32Dtetp210Bcr-Abl Cells

In Fig. 4A, we present the structures of the furans that were synthesized by the reaction shown in Fig. 1A. The initial analysis suggested a role for the amide group in the suppression of the growth of the 32Dtetp210Bcr-Abl cell line. These data suggested that a bulky group in *cis* to the amide on the furan ring could reduce its inhibitory effect. A series of furans was then synthesized in which the steric effects of groups neighboring the amide were minimized (see Fig. 4A).

These compounds completely suppressed the growth of the 32Dtetp210Bcr-Abl cell line (see Fig. 4B) at nanomolar concentrations in the low-density cell proliferation assay. Furthermore, their effect at the 10 nmol/L level was almost totally selective for the Bcr-Abl–expressing cell line because no growth suppression was seen on the 32D cell line in the presence of IL-3.

In-Gel Kinase Cell-Free Assay of Inhibition of Src and v-Abl Kinase Proteins by the Acetylenes and Furans

The cell-free inhibitory effects of the acetylene and furan compounds on the Src protein kinase in an ''in-gel'' kinase assay are shown in Fig. 5A. The addition of the furan compounds A101 and A103 and the acetylene AC22 individually suppressed the Src protein kinase activity by 1.5- to 2-fold. These assays suggested that AC22 acetylenic compounds as well as the furan compounds A101 and A103 possess inhibitory activity against the Src protein kinase.

The A102, A103, A104, and A105 furan compounds produced at least a 5-fold decrease in v-Abl protein kinase activity, whereas A101 led to an ~2-fold decrease (Fig. 5B). These results suggest that the inhibitory effects of the furan compounds are greater on the v-Abl than on the Src protein kinase.

Inhibitory Effect of the Acetylene Compounds on the Growth of Leukemia/Lymphoma and Solid Tumor Cell Lines

K1P, AC19, and AC22 were sent to the National Cancer Institute for an additional screening of a panel of 60 established cell lines derived from patients with leukemia, lymphoma, or solid tumors. The data for cases in which the inhibitory effect on the cell lines is measurable in the leukemic panel are presented in Table 3. K1P and AC22 inhibited the growth of cell lines derived from myeloid and lymphoid acute and chronic leukemias, multiple myeloma, non–Hodgkin's lymphoma, as well as colon and renal cancer cell lines.

Inhibitory Effect of AC22 and K1P on a Panel of 60 Kinases

To formally test the hypothesis that the acetylenic compounds could be also acting at another target in addition to p210Bcr-Abl, AC22 and K1P were tested for their inhibitory activity on 60 kinases through a contract firm, Upstate Biotechnology. At 10 μmol/L, AC22 almost completely inhibited the activity of the Src-related protein kinase Lyn and K1P inhibited Fyn, another Src family kinase (see Table 4).

Discussion

Two groups of compounds have been identified, which inhibit proliferation of the 32Dtetp210Bcr-Abl cell line under conditions in which cell growth is dependent on the Bcr-Abl protein kinase, in the absence of added growth factors, such as IL-3. When the inhibitory effects of acetylene-derived lead drugs (AC22, AC19, and K1P) on the growth of the 32Dtetp210Bcr-Abl cell line were tested in the absence of serum in the low-density cell growth inhibition assay, significant inhibition was seen at the

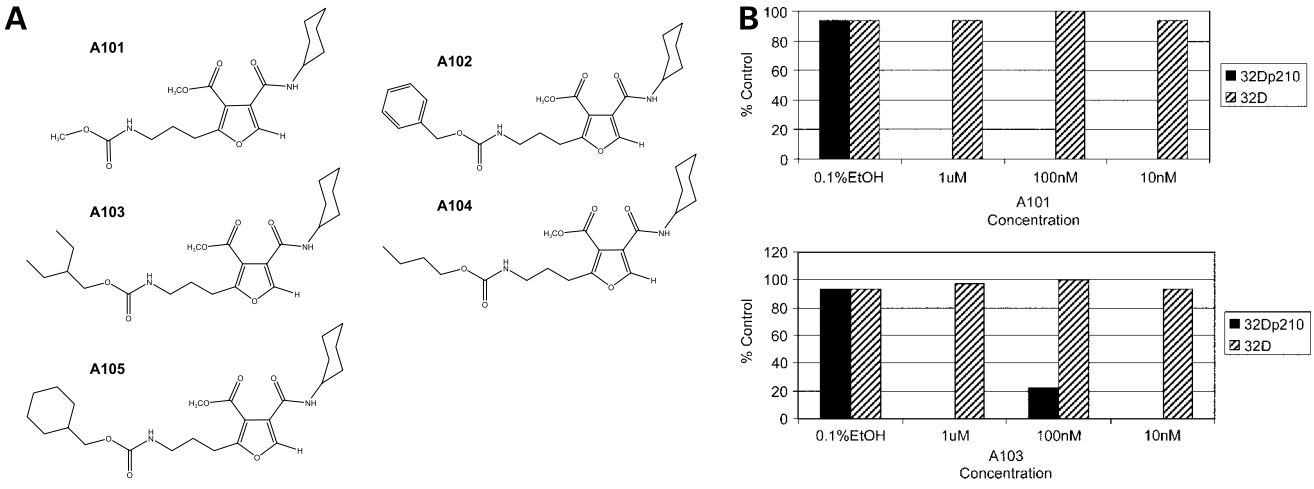

Figure 4. **A,** structure of the furans in which steric factors near the amide are minimized. Based on the early studies in which the role of the size of the chemical functionality in *cis* to the amide, a series of compounds was made in which only a hydrogen atom rather than a methyl group or larger in *cis* to the amide was created. Each of the compounds tested showed significant suppression of the growth in the low-density cell proliferation assay of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L level. As shown, the action of these drugs was almost totally selective for the p210Bcr-Abl – dependent growth. **B,** inhibitory effect of the furans on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. The low-density cell proliferation assay was used to study the suppression of the growth of the 32Dtetp210Bcr-Abl cell line following addition of the A101 and A103 furans.

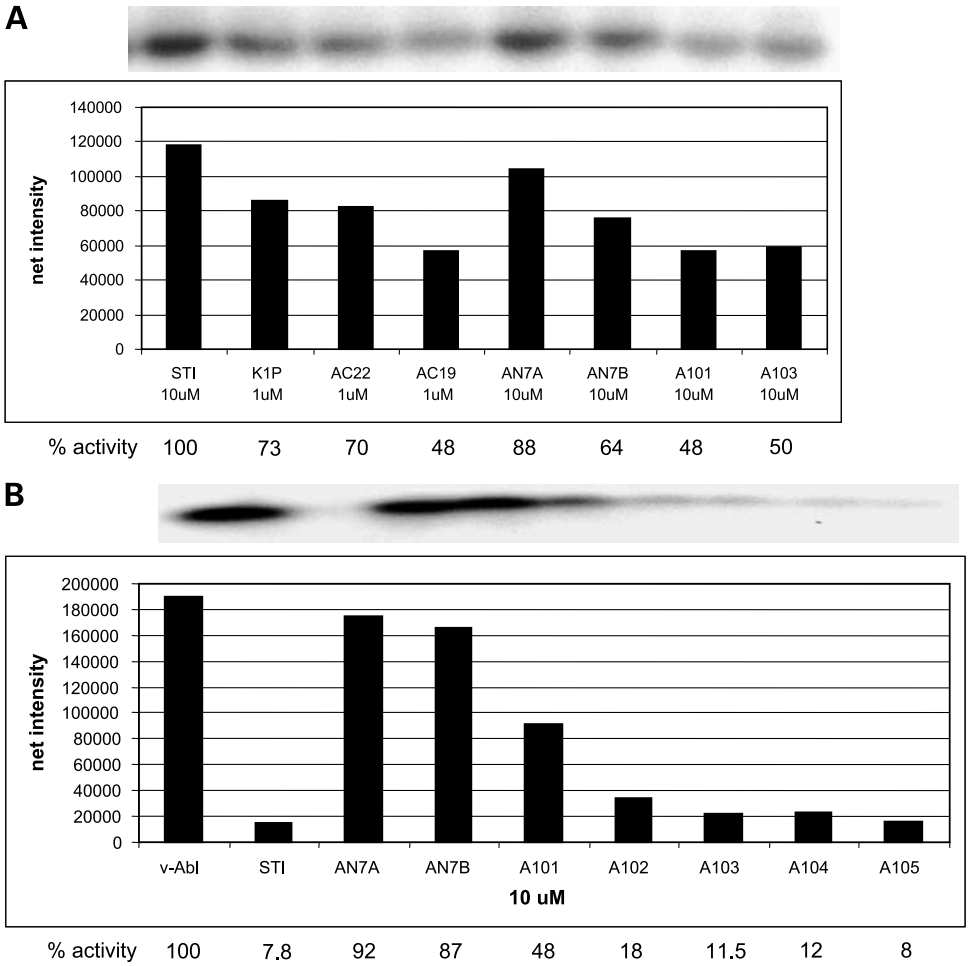

Figure 5. *In vitro* effect of furans and acetylenes on Src (**A**) and v-Abl (**B**) kinase activity in cell-free assay. The recombinant purified enzyme and its substrate following exposure to each of the test drugs were added in the presence of [γ-^{32}P]ATP to the sample well of a SDS-PAGE. Following the completion of the electrophoresis, the gel was autoradiographed with substrate for the Src kinase (**A**) and the v-Abl kinase (**B**). **A,** effects of the K1P, AC22, and AC19 acetylenic compounds as well as the furan compounds AN7A, AN7B, A101, and A103 on the Src kinase. **B,** effects of the furan compounds AN7A, AN7B, A101, A102, A103, A104, and A105 on the v-Abl kinase. *STI,* imatinib.

100 nmol/L level. When the same drugs were tested for suppression of growth of the 32Dtetp210Bcr-Abl cell line at high cell density (using MTS) in the presence of serum, the concentration of drugs required for significant growth inhibition is in the 1 μmol/L range.

Interestingly, when individual acetylene compounds AC22 and K1P are combined with imatinib in the absence of serum, they suppress the proliferation of the 32Dtetp210Bcr-Abl cells at the 10 nmol/L range. In addition, the inhibitory activity of the combination of AC22 or K1P with imatinib is extremely selective for p210Bcr-Abl–dependent growth because no inhibition is seen on the 32D cell line in the low-density cell proliferation assay. Similarly, the furan-derived compounds presented in Fig. 4 are also extremely selective and potent inhibitors of the growth of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L concentration level.

We also studied the effect of the acetylenes on the growth of two imatinib-resistant cell lines that overexpress Bcr-Abl. These compounds showed a higher inhibitory effect on the Baf/*BCR-ABL*-r than on the LAMA84-r cell line. This may be due to the fact that, in addition to Bcr-Abl overexpression, LAMA84-r, but not Baf/*BCR-ABL*-r, also overexpresses the *multidrug resistance 1* P-glycoprotein. Whether the acetylene compounds are substrates of this pump protein has not yet been proven. In future work, it will be also important to test the inhibitory effect of the furans and acetylenic compounds on cell lines in which the resistance to imatinib is due to the acquisition of point mutations in the p210Bcr-Abl kinase. This latter mechanism is the most common mechanism through which imatinib resistance is acquired in patients (7).

It is remarkable that two classes of such good compounds were identified from so few candidates. Our screening strategy was designed to identify compounds that would inhibit proliferation of the 32Dtetp210Bcr-Abl protein kinase-dependent cells at any point downstream of the complex pathways emanating from the action of the p210Bcr-Abl kinase. It is thus possible that this strategy increased the chances of finding compounds that were inhibitory to imatinib-resistant cell lines.

Imatinib was shown early on to suppress the level of the circulating myeloid cell mass in most patients with chronic-phase CML and in a lower fraction of patients with accelerated- or acute-phase CML. At least 24 different point mutations of the p210Bcr-Abl tyrosine-specific

Table 3. Results from the National Cancer Institute screening of growth inhibition of a panel of leukemia and solid tumor cell lines with 1 μmol/L of test drug in a high-density cell proliferation assay

Cell line	% Cell growth inhibition by 1 μmol/L of test drug					
	From	K1P	AC22	AC19	K11P	Cell no.
HL-60 (TB)	PML	75	81	96	88	40,000
K-562	CML-EL	80	96	101	103	5,000
MOLT-4	ALL-T	50	78	104	66	30,000
RPMI-8226	MM*	79	77	80	84	20,000
CCRF-CEM	ALL-T	—	130	101	—	40,000
SR	NHL	—	65	96	—	20,000

Abbreviations: PML, promyelocytic leukemia; ALL, acute lymphocytic leukemia; MM, multiple myeloma; NHL, non–Hodgkin's lymphoma.
*Resistant to dexamethasone.

protein kinase were found to be associated with the acquisition of resistance to imatinib (19). In addition, other mechanisms of resistance were found other than point mutations, such as overexpression of the P-glycoprotein. The point mutations were found in a few patients with primary resistance to imatinib at diagnosis and in a much larger number of patients with acquired resistance at the time of emergence of resistance to imatinib in accelerated- and acute-phase patients.

Thus, a major priority was to find additional drugs that could be used to treat CML patients in whom resistance to imatinib was acquired or was present at diagnosis. Several drugs have been reported to suppress populations of CML cells that display resistance to imatinib (20–23). Both preclinical and clinical data are available on drugs that inhibit the p210Bcr-Abl kinase both in the activated and in the inactivated states (24). Clinical trial results of dasatinib (BMS 354825) have induced complete hematologic and cytogenetic responses in imatinib-resistant Philadelphia chromosome–positive leukemias (23). Unlike imatinib, this drug binds both the active and the inactive conformations of the p210Bcr-Abl protein (24).

A phase I trial showed that nilotinib (AMN107) induced hematologic responses in 13 of 33 and 9 of 33 cytogenetic responses in blast crisis patients (25). In accelerated-phase patients, 33 of 46 patients developed hematologic responses and 22 of 46 developed cytogenetic responses (25). Nilotinib is 10- to 50-fold more potent than imatinib in preclinical models (26, 27). A new non–ATP-competitive inhibitor (ON012380) has been shown to inhibit 100% of the mutations, which lead to imatinib-resistant CML cells, including the T3151 mutation, at the 10 nmol/L level (28). PD166326, which belongs to a family of pyridopyrimidines, inhibits *in vivo* growth of imatinib-resistant p210Bcr-Abl–positive cells in preclinical models at the nanomolar concentration (29). Eventually, such drugs may be combined at diagnosis with imatinib to reduce the probability of evolution of imatinib resistance, especially in patients with accelerated and acute phases of CML or in chronic-phase CML patients with adverse clinical features who are therefore considered to be at increased probability of evolution of resistance to imatinib.

There are several unique features of the drugs reported in this article that distinguish them from the other CML inhibitory drugs already reported (19–29), which suggest that they may play a unique clinical role: (*a*) the acetylene AC22 has been shown not only to suppress the growth of CML cells, which are resistant to imatinib, but also to inhibit p210Bcr-Abl–dependent cell growth at the nanomolar concentrations when combined with imatinib; (*b*) the level of inhibition of the combination of AC22 and imatinib is greater than the sum of the inhibitory effects of AC22 and imatinib when used separately; (*c*) the combination of AC22 and imatinib is totally noninhibitory to the non–p210Bcr-Abl-dependent cell growth at the 10 nmol/L level; (*d*) the acetylenes AC22 and K1P are inhibitory to imatinib-resistant cells; (*e*) the furans (e.g., A103 and A105) are inhibitory to p210Bcr-Abl–dependent cell growth at the 10 nmol/L concentration at which these drugs do not inhibit cell growth that is not p210Bcr-Abl dependent; and (*f*) the acetylene AC19 is inhibitory to p210Bcr-Abl–dependent cell growth at the 1 μmol/L level, at which level the drug is totally noninhibitory to the p210Bcr-Abl–independent cell growth.

The apparent synergy of AC22 with imatinib is unique among all of the classes of drugs tested thus far (19–30).

Table 4. Screening of a panel of 60 kinases for inhibition of enzymatic activity in the presence of test drug in cell-free assay

	% Inhibition at 10 μmol/L*	
	K1P	AC22
CaMKII	—	40
CaMKIV	—	64
CDK2/cyclinA	—	50
CK1	46	(37)
Fyn	49	(29)
IKKβ	—	45
Lyn	—	98
PKCy	—	61
PKCbII	(35)	48

*Data are presented only for kinases for which the inhibition activity is >45%.

In addition, the selectivity of the furans A103 and A105 at the 1 nmol/L level seems to also be unique among all of the classes of drugs thus far developed. In addition, the experimental results suggest that these drugs may be useful for the treatment of patients in whom resistance to imatinib has already emerged.

Our studies showed that the acetylenic compounds K1P and AC22 also inhibit Src and the Src-related kinases Lyn and Fyn. This is a very interesting result in light of the recent report that the Lyn protein kinase is overexpressed in imatinib-resistant cell lines K-562-R (9). The Lyn kinase has been reported to be involved in the response of the cell to DNA-damaging agents, such as the Abl kinase (9, 10, 30). This suggests that these compounds might be acting at multiple different targets downstream of p210Bcr-Abl.

In addition, the use of a synthetic strategy to generate the low-complexity combinatorial libraries of compounds that maintains the chirality of the chemical functionalities on the two types of molecular scaffolds (linear-acetylenes or planar cyclic-furans) may have reduced the number of candidates needed to identify potent and selective inhibitors of the 32Dtetp210Bcr-Abl cell line.

Another remarkable feature that was observed among the acetylene-derived compounds from our libraries is that some of them suppress imatinib-resistant cell lines. Such compounds could conceivably be of value when used together with imatinib for the initial treatment of CML because the probability of the evolution of resistance to a combination might be lower than with single-agent therapy.

Further studies are required to characterize these compounds at the preclinical level before they can be considered for introduction into the clinic. The toxicity and bioavailability of each of these compounds are undergoing study in additional cell lines and, more importantly, in primary leukemic cells. Both the furans and the acetylenes have multiple sites at which additional functional groups can be added to increase bioavailability and that can improve the pharmacokinetics. It is envisaged that the ongoing systematic study of these compounds in animal models of Bcr-Abl–induced leukemia will help us optimize their pharmacology.

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